STUDIES ON THE TRANSGLUCOSIDATION OF SCHIZOSACCHAROMYCES POMBE (Part 2) ISOLATION AND IDENTIFICATION OF THE SYNTHESIZED OLIGOSACCHARIDES

STUDIES ON THE TRANSGLUCOSIDATION OF *SCHIZOSACCHAROMYCES POMBE* (Part 2)*
ISOLATION AND IDENTIFICATION OF THE SYNTHESIZED OLIGOSACCHARIDES

By

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(*Received June 10, 1955*)

Previously (1), it was demonstrated by paper chromatography that *Schizosaccharomyces Pombe* synthesized sakébiose, kojibiose, isomaltose, panose and isomaltotriose from maltose and sakébiose, kojibiose and isomaltose from glucose. Herein I wish to report on the isolation and identification of those synthesized oligosaccharides. Previously, maltose was used as the substrate of transglucosidation but in this report the starch sirup which contained glucose and several members of the homologous series of 1,4-α-linked glucose polymers, namely, maltose, maltotriose, maltotetraose and maltopentaose was used. In the next communication I will report on the identification of those oligosaccharides contained in the starch sirup.

It was considered those oligosaccharides were synthesized mainly from maltose which was hydrolyzed from maltotriose, maltotetraose and maltopentaose etc. and partly from glucose.

Experimental

(I) Synthesis of Oligosaccharides

Washed cells (62 g) of *Schizo. Pombe*, which had been grown on a Kôji-extract (Bollg. 10, pH=5.0) were suspended in 1.5 L of a solution containing 200 g of starch sirup. The resulting suspension was incubated for two hours at 30°C. The composition of sugars of starch sirup which contained glucose and several members of the homologous series of 1,4-α-linked glucose polymers was analyzed by the paper chromatographic method described by Flood, et al (2), as shown in Table 1.

* Studies on the Unfermentable Sugars (XV).
The Original Japanease Report (Journal of the Agricultural Chemical Society of Japan Vol. 29, 1955).

Table 1. Composition of sugars of sirup by paper chromatograhpic analysis

Component	per cent	per cent for total sugar
Glucose	10.69	11.60
Maltose	20.95	22.73
Maltotriose	20.95	22.73
Maltotetraose	10.85	11.77
Maltopentaose	6.57	7.13
Higher Oligosaccharides	22.15	24.03
Total	92.16	99.99

Total sugar = 92.17%
Reducing sugar = 38.44%

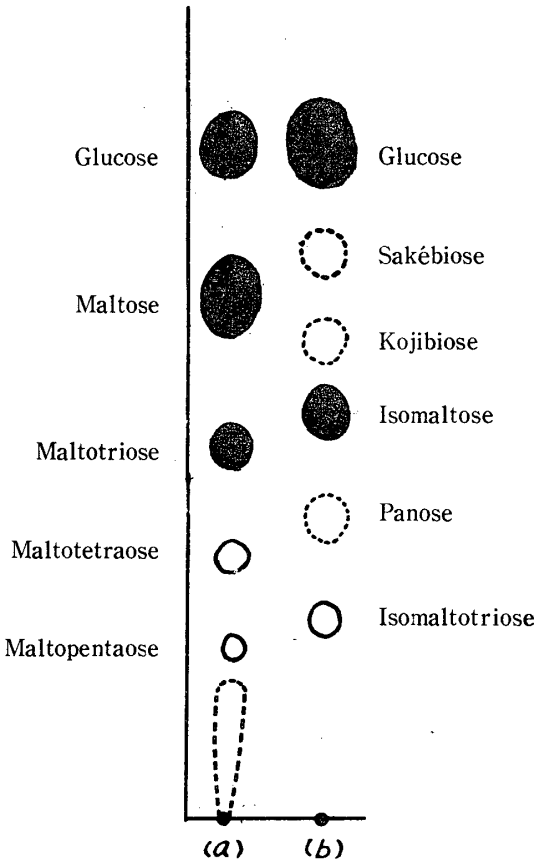

Fig. 1. Multiple paper chromatograms of oligosaccharides
(a) Starch sirup which was used as a substrate
(b) Digestion of Starch sirup by *Schizo. Pombe*

The four times multiple chromatogram of starch sirup is shown in Fig. 1(a). The method used in our laboratory for the identification of sugars by paper chromatography was described previously (3). For the elution solvent, a mixture of pyridine, butanol and water (2 : 3 : 1.5, respectively) was used. For the developing agent, aniline hydrogenphtalate was used.

After incubation, the cells of *Schizo. Pombe* were removed in the centrifuge. The oligosaccharides in the supernatant liquid, which were synthesized by *Schizo. Pombe*, were examined on a paper chromatography. The obtained four times multiple chromatogram are shown in Fig. 1(b). The elution solvent and developing agent were the same as previously described. The composition of sugars of the supernatant liquid was analyzed by the paper chromatographic method. Namely, the respective spots of sugars on the four times multiple chromatogram were cut off by the guide strips and then extracted with water. The extracted sugars were analyzed using the method of Stark and Somogyi(4).

The obtained analytical data of the composition of the synthesized oligosaccharides are shown in Table 2 (A) and (B).

Table 2 (A) Analysis of oligosaccharides synthesized from sirup

Component	per cent	per cent for total sugar
Glucose	5.18	47.27
Sakébiose	0.28	2.60
Kojibiose	0.44	4.03
Isomaltose	1.25	11.40
Panose	0.33	3.01
Isomaltotriose	0.40	3.65
Higher oligosaccharides	3.07	28.04
Total	10.95	100.00

(B) Concentration of sugar

Per cent of	Initial solution	Solution after fermentation
Total sugar	14.10	10.95
Reducing sugar	5.64	6.27

From the above results, it was demonstrated that several members of the homologous series of 1,4-α-linked glucose polymers, namely, maltose, maltotriose, maltotetraose and maltopentaose were hydrolyzed or transglucosidated and sakébiose, kojibiose, isomaltose, panose, isomaltotriose which were not contained in the original starch sirup were synthesized. Those synthesized oligosaccharides on paper chromatograms were examined by the technique of a mixed chromatography with the pure sugars (R_f value, color, overlap with two sugars and etc.).

(2) Isolation of the Synthesized Oligosaccharides

I also tried to isolate and identify those synthesized oligosaccharides as crystalline substances. The supernatant liquid of the digestion was neutralized, vaparized alcohol by distilation under reduced pressure and fractionated on a charcoal column by Whistler and Durso's method(5) which involved the passage of the solution through a charcoal column and subsequent elution of the column with aqueous solution containing increasing concentrations of ethanol.

The column was prepared in a glass cylinder measuring 55×12 cm with a fritted glass bottom. The adsorbent was made by mechanically mixing 780 g of active carbon (Takeda) with Celite No. 545. 1.5 Liter of the supernatant liquid was poured into the cylinder and sufficient suction was applied to draw the liquids through at a rate of 1 liter per hour. The effluent containing the desorbed material was caught at the bottom of the unit in a large suction flask in 3 liter quantities. Each 2 liter of eluates of water with the exception of

the first, was concentrated in vacuo to a small volume. A large quantity of sirup was obtained from the I st~II nd eluates; paper chromatography revealed glucose to be the only constituent. The V th~VI th eluates were proved to contain only a small quantity of isomaltose. As it was a small quantity of sirup, elution with aqueous solution was changed to 5 per cent ethanol solution. From these effluents isomaltose and kojibiose were revealed. The concentrations of ethanol was changed to 10, 15 and 25 per cent step by step. And about 130 L of effluentes were obtained, each portion being kept separate. In such a way, each 2 liter of eluates were separately concentrated. The number of sugars in each was roughly determined by chromatography on paper strips. The fractions which several sugars were mixed, were further purified by rechromatography. The obtained fractions are shown in Table 3.

Table 3. Fractions of effluents by carbon column chromatography

Fraction No.	Sugar component by paper chromatography	Solvent used for elution
1	Glucose	Water
2	Isomaltose	"
3	Isomaltose, Kojibiose	5 per cent ethanol
4	Sakébiose, Maltose	"
5	Maltotriose	"
6	Panose	10 per cent ethanol
7	Isomaltotriose, Kojitriose	"

(3) Identification of the Synthesized Oligosaccharides

Each of the sirups was concentrated under reduced pressure and further dried up. This substance was disolved in hot methanol and the insoluble materials, for example Celite etc. were filtered off. The filtrate was removed of the solvent by evaporation under reduced pressure and dried up by distilation with methanol under reduced pressure into a white amorphous powder. The amorphous powder was acetylated with newly fused sodium acetate and acetic anhydride at 100~105°C for two hours. The resulting acetylated material was poured into ice water. After the hydrolysis of residual acetic anhydride, the sugar acetate was extracted with chroloform. The extract was washed with an aqueous solution of sodium carbonate and subsequently with water. After removal of the solvent by evaporation under reduced pressure, the amorphous material was dried in a vacuum desicator and then crystallized from 95 per cent ethanol at various temperatures.

(A) Glucose—Fraction:

The amorphous material of Fraction 1 (in Table 3) was dissolved in a small quantity of hot water and placed in an ice box overnight. A heavy crop of sugar was obtained. This was separated by filtration, washed, and after

air-drying it was recrystallized from 95 per cent hot ethanol and identified as *D*-glucose monohydrate, MP 81.0~81.5°C. 5 g of this crystalline *D*-glucose was acetylated by the method as described above. 6.8 g of the crystalline material was obtained after recrystallization from ethanol. MP 129~130°C unchanged on admixture with authentic β-*D*-glucopyranose-pentaacetate (MP 129~130°C).

The sugar in Fraction 1 was identified as *D*-glucose by means of its crystalline acetic derivative.

(B) Isomaltose—Fraction :

The amorphous material of Fraction 2 (in Table 3) is very hygroscopic. 5 g of the material was acetylated by the method as described above. 10.1 g of the amorphous acetate was obtained and crystallized from 95 per cent hot ethanol at 20~25°C. The crystalline material was disolved in 95 per cent hot ethanol and to it was added active carbon. After filtration of active carbon, it was further purified by three times recrystallization from ethanol at room temperature. 6.0 g of well-buillt crystals (prism) were obtained. MP 143~144°C unchanged on admixture with β-isomaltose-octaacetate (MP 143~144°C), for this, I am idedted to Dr. Allene Jeanes.

The sugar in Fraction 2 was identified as isomaltose by means its crystalline acetic derivative.

In an attempt to crystallize free isomaltose, 5 g of the crystalline β-isomaltose octaacetate was deacetylated according to the method of Thompson (6). The acetate was disolved in 50 ml of methanol and cooled to 0°C. Sodium methylate (2.5 ml of 1 N) was added to the solution and the whole kept at 0°C for twenty-four hours. It was then diluted with 200 ml of cold water and the ionic materials were removed by passing over Amberite resins IR-120 and IR-410. The effluent from the ion exchange columns was concentrated to a sirup under reduced pressure and further dried up. This amorphous material was disolved in hot methanol and the insolble materials were filtered off. The filtrate was removed of the solvent by evaporation under reduced pressure and dried by distillation with methanol under reduced pressure into a white amorphous powder. It is a very hygroscopic solid like the original crude isomaltose. Only one spot appeared on the paper chromatogram. Comparative tests with isomaltose which was indebted Dr. Allene Jeanes by the technique of a mixed chromatography showed it was isomaltose. All attempts to crystallize the substance failed.

(C) Kojibiose, Isomaltose—Fraction :

From this fraction two spots appeared on the paper chromatogram. One of them was isomaltose. Comparative tests with kojibiose which was isolated from Kôji-extract and considered to be 2-*O*-α-*D*-glucopyranosyl-*D*-glucopyranose(7), by the technique of the mixed chromatography (R_f and pink or reddish brown color by aniline hydrogenphtalate), showed that another spot sugar was

kojibiose.

The R_f value of kojibiose agreed with the three R_f values of cellobiose, α,α- and α,β-trehalose even through three times multiple chromatogram. But kojibiose has a color in the spot different from cellobiose which showed a brown color and did not hydrolyzed by emulsin whereas cellobiose did. Nonreducing trehalose gives a distinct spot only by ammonical silver nitrate, but not by aniline hydrogenphtalate. The spots of those four sugars which have the same R_f value as above mentioned were separated distinctly on the chromatogram of the sugar-borate paper ionophoresis and showed the different Mg value 0.31 (kojibiose), 0.27 (cellobiose) and 0.10 (α,α- and α,β-trehalose) respectively(8).

From the above mentioned experiments, the other sugar in the Fraction 3 (in Table 3) was considered to be kojibiose.

Acetylation: The R_f value of kojibiose is in close agreement with that of isomaltose. Kojibiose could not be isolated from isomaltose by two or three times carbon column rechromatography. Thus, kojibiose was purified as acetyl derivative.

The amorphous material was acetylated in the manner just described. The acetylated material was disolved in 95 per cent hot ethanol and placed in a thermostat at 20~25°C overnight. A heavy crop of acetate was obtained. This was separated by filtration, washed and after air-drying it recrystallized from 95 per cent hot ethanol and was identified as β-isomaltose octaactate, MP 143~144°C. All attempts to crystallize kojibiose-octaacetate from the filtrate failed. After removal of the solvent, the amorphous material was chromatographed on Magnesol-Celite by the method described by Wolfrom et al (9): benzene and t-butyl alcohol was used as the developing agent. One zone appeared on the column. The zone material was removed from the sectioned column by elution with acetone. After removal of the acetone the zone failed to crystallized.

(D) Sakébiose, Maltose--Fraction:

4.1 g of the amorphous material of Fraction 4 (in Table 3) was acetylated in the manner just described above. 7.7 g of the amorphous acetate was obtained and crystallized from 95 per cent hot ethanol. After 5~6 times recrystallization, 1.7 g of the crystalline material in the form of a very long thin prism was obtained: MP 158°C unchanged on admixture with authentic β-maltose octaacetate (MP 159°C). Soon after filtration of the crystalline substance of β-maltose octaacetate, the new crystalline material was obtained and was further purified by 10 times recrystallization from ethanol: the pure crystalline substance weighed 1.5 grames; MP 150~150.5°C unchanged on admixture with sakébiose-octaacetate which was isolated from koji-extract and identified as crystalline octaacetate of 3-*O*-α-*D*-glucopyranosyl-*D*-glucopyranose

(MP 150~150.5°C).

(E) Maltotriose—Fraction :

From this fraction one spot appeared on the paper chromatogram which showed a little smaller R_f value than that of isomaltose and a greenish brown color as 1,4-α-linked polymer(10). It semed to be maltotriose.

1.9 g of the amorphous material was acetylated in the manner described above. 3.1 g of the amorphous acetate was obtained and dissolved in 95 per cent hot ethanol and to it was added a sufficient amount of active carbon. After filtration of active carbon the filtrate was placed in a thermostat at 33~35°C overnight. The obtained crystalline substances were further purified by three recrystallizations. 1.5 g of the crystalline material in the form of a long prism was obtained. MP 132°C unchanged on admixture with β-maltotriose hendecaacetate (MP 132°C) for this I am indebted to Profesor M. L. Wolfrom.

The sugar of the spot which appeared on the paper chromatogram of Fraction 5 was maltotriose and probably not synthesized sugar by *Schizo. Pombe* but it might be a residual sugar of the substrate.

(F) Panose—Fraction :

From this fraction one spot appeared on the paper chromatogram which showed a little smaller R_f value than that of maltotriose and the same greenish brown color as 1,4-α-linked polymer. Comparative tests with panose (due to the kindness of Profesor M. L. Wolfrom), by a techhique of a mixed chromatography (R_f, overlapping of two sugars and color etc.), showed that the sugar of the spot was panose.

The amorphous materials were difficult to dissolve in methanol. Therefore 1.5 g of the amorphous materials were dissolved in 1 ml of hot water to which 6 ml of methanol was added slowly with stirring. Any turbidity that formed during the addition of methanol were completely removed by centrifugation. The clarified aqueous methanol solution was kept in a tightly colsed flask at 20~25°C for 1~2 days. The obtained crystals were collected by filtration, washed with methanol and further purified by three recrystalizations from aqueous methanol : MP 215°C (thin prism), unchanged on admixture with panose (for wich I am indebted to Profesor M. L. Wolfrom). The sugar in the Fraction 6 was identified to be panose which was composed of three glucose units linked by a 1,4-α and a 1,6-α-linkage and was 4-α-isomaltopyranosyl-*D*-glucose.

(G) Isomaltotriose, Kojitriose—Fraction :

From this fraction one spot appeared on the paper chromatogram which showed a little smaller R_f value than that of panose and the dark brown color as 1,6-α-linked polymer. Comparative tests with isomaltotriose (the material was received from Dr. Allene Jeanes), by a technique of a mixed chromato-

graphy, showed that the sugar of the spot was isomaltotriose. Then by three to four times multiple paper chromatography the new spot with showed a very little higher R_f value than that of isomaltotriose and the pink or reddish brown color the same as kojibiose appeared. The relation between isomaltotriose and this new sugar closely resembles that of isomaltose and kojibiose on a paper chromatography, carbon column chromatography and sugar borate paper ionophoresis etc.

Since it is difficult to isolate these sugars, the respective spots of sugars on the four times multiple paper chromatogram were cut off by guide strips and then extracted with water. Each extracted sugar solution was concentrated to a thick sirup under reduced pressure and further dried by distillation with methanol under reduced pressure.

To the amorphous material which was obtained from the spots of the dark brown color was added 0.2 N H_2SO_4, hydrolyzed for 60 min. on the steam bath, and then neutralized with $Ba(OH)_2$. After removal of $BaSO_4$ by centrifugation, the supernatant liquid was examined by paper chromatography. Namely, three spots appeared and they were identified as glucose, isomaltose and residual isomaltotriose. Furthermore the amorphous material was hydrolyzed by Taka-diastase for 7~24 hours at 55°C. The spots of glucose, isomaltose, isomaltotriose and higher oligosaccharide appeared on the paper chromatogram.

From the above mentioned experiments, one of the sugars in the Fraction 7 was considered to be isomaltotriose which was composed of three glucose units linked by two 1,6-α-linkage and was *O*-α-*D*-glucopyranosyl-(1→6)-*O*-α-*D*-glucopyranosyl-(1→6)-α-*D*-glucopyranose.

The other sugar which showed a pink or reddish brown color in the spots of a paper chromatogram was more difficult to hydrolyze than isomaltotriose. To the amorphous material was added 0.2N H_2SO_4, hydrolyzed for three hours on the steam bath and neutralized with $Ba(OH)_2$. After removal of $BaSO_4$ by centrifugation, the supernatant liquid was examined by a paper chromatography. Namely, three spots appeared and they were identified as glucose, kojibiose and residual sugar. When they were hydrolyzed completely, paper chromatography revealed glucose to be the only constituent.

On the other hand, the regularity of the papergram mobilities of the homologous series of carbohydrates was demonstrated by Jeanes, French and Aso et al (11, 12, 10,). Aso et al have found a linear relationship between logarithm of a partition function α' which was defined as $\log\left(\frac{R_f}{1-R_f}\right)$ and the molecular size of the homologous oligosaccharides series. Then $\log \alpha'$ of glucose, kojibiose and the unknown sugar formed straight line.

From the above mentioned experiments, the other unidentified sugar was considered to be composed of three glucose units linked by two 1,2-α-linkage

and was designated herein as "kojitriose".

(H) Blank test:

A large quantity of cells of *Schizo. Pombe* were suspended in a sirup solution, so that many synthesized oligosaccharides may be derived from the constituents of the cells. Further experiment was continued as follows. 50 g of the cells of *Schizo. Pombe* were suspended in one liter of water without adding starch sirup. The suspension was incubated for two hours at 30°C in the manner as described above. After removal of the cells in the centrifuge, the supernatant liquid was concentrated to 5 ml under reduced pressure. There was no such sugar as oligosaccharides in the concentration by paper chromatography. It was demonstrated that sakébiose, kojibiose. isomaltose, panose, isomaltotriose and kojitriose were synthesized from the several members of the homologous series of 1,4-α-linked glucose polymers.

Summary

I studied the synthesis of oligosaccharides by *Schizosaccaromyces Pombe* from the sirup which contained glucose and several members of the homologous series of 1,4-α-linked glucose polymers. The washed cells of *Schizo. Pombe* were suspended in the sugar solution (Total sugar 14.10 per cent, Reducing sugar 5.64 per cent) and the mixture was incubated for two hours at 30°C. It was demonstrated by paper chromatography that *Schizo. Pombe* synthesized sakébiose, kojibiose, isomaltose, panose, isomaltotriose and kojitriose. The digest was fractionated by carbon column chromatography. Those oligosaccharides obtained from each fractions were identified as crystalline acetates or free sugar.

The new trisaccharide which probably consisted of the two 1,2-α-glucosidic linkages herein designated as Kojitriose, was isolated from the digest.

Acknowledgments

I wish to express my thanks to Profesor Kiyoshi Aso for his kind advice, to the Nagao Institute for providing the strains of *Schizosaccharomyces Pombe*, to Profesor M. L. Wolfrom and Dr. Allene Jeanes for providing many oligosaccharides.

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